Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Glocker E-O, Hennigs A, Nabavi M, et al. A homozygous *CARD9* mutation in a family with susceptibility to fungal infections. N Engl J Med 2009;361:1727-35.

Supplemental Appendix

(Citations: all citations refer to the reference list in the primary manuscript document.)

Content

1. Supplementary methods and results

Methods

Single nucleotide polymorphism (SNP) genotyping and analysis

Genomic DNA was digested with the restriction enzyme *Nsp*I, ligated with an adaptor and subsequently amplified using a single adaptor primer to yield PCR fragments in the size range of 250 bp -1100 bp. Purified PCR fragments were then fragmented, labeled and hybridized to the array for 16h. After washing and staining, the arrays were scanned using an Affymetrix GeneChip Scanner 3000 G. Genotyping Console 2.0 software from Affymetrix was used for further analysis (http://www.affymetrix.com/support/technical/whitepapers/brlmm_whitepaper.pdf).

SNP genotypes were further analyzed using in-house software to identify intervals where the affected individuals are homozygous for the same genotypes at consecutive markers, and the unaffected individuals have different genotypes for at least some markers in the interval. The software implements a two-state finite automaton to keep track of whether the current SNP is in a run where all affected are homozygous or not; when in a homozygous run, a count is kept of how many SNPs have a different genotype in each unaffected individual. Because the state changes based only on the affected individual genotypes, one or more unaffected individuals

being homozygous at a marker cannot create a boundary for an interval consistent with linkage. This cautious rule was chosen because the SNP genotypes may have errors and the disease penetrance might be incomplete.

Microsatellite genotyping and analysis

LOD scores shown here were computed assuming full penetrance for individuals homozygous for the putative disease-associated mutation and a phenocopy rate of .5% for the other two possible genotypes (individuals are the result of phenocopies if they show a similar phenotype to the disease phenotype under study, but actually lack the responsible genotype; e. g. in our study, patients with candida due to AIDS would have been results of phenocopies); in LINKAGE notation, the penetrance function was 0.005 0.005 1.000. The disease-allele frequency was set to 0.001. Marker allele frequencies were set all equal due to the limited number of samples. The phenocopy rate was set to be non-zero because: a) fungal infections are not uncommon and b) analysis of the SNP data suggested that one of the affected individuals is a phenocopy. To check robustness, the LOD score calculations were also redone with a variety of similar models and these had only a small effect (change of at most 0.1) on the positive scores (data not shown).

DNA extraction, PCR and sequencing

DNA of study participants was isolated by using the Gentra Puregene Blood kit (Qiagen, UK) according to manufacturer's instructions. Coding regions of *CARD9* (Ensembl No. ENSG00000187796) were amplified on a Techne 412 Thermocycler (Techne, UK) by using a standard protocol (all primers were purchased from Invitrogen, UK; primer sequences are available on request). Amplicons were examined by applying 5 µL on a 1.2% agarose gel (Peqlab, Germany) and then

purified using the QIAquick PCR purification kit (Qiagen, UK). Purified PCR products were sequenced with the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit v3.1 (Applied Biosystems, UK) using the PCR primers as sequencing primers. Sequencing was accomplished with an ABI 3130xl DNA Sequencer (Applied Biosystems, UK); the sequence reads were analyzed with Sequencing Analysis software and the Sequencher® software (Genecodes, US).

RNA isolation and quantification of CARD9 expression

A 174 bp fragment of *HPRT1* (Hypoxanthine-guanine phosphoribosyltransferase 1; using the primers HPRT1-Fw 5-AGC CAG ACT TTG TTG GAT TT-3 and HPRT1-Rv 5-AGG ACT CCA GAT GTT TCC AA-3) and a 1799 bp fragment of *CARD9* (using the primers CARD9-Fw 5-CCT GGT GTG TCT GCA GTG-3 and CARD9-Rv 5-GCA CCA GAT TCC TCG TTC CAG-3) were amplified by conventional PCR using complementary DNA (cDNA) of a healthy individual as template. After examination and purification, amplicons were ligated into a pGEMeasy vector (Promega, UK) followed by transformation of JM109 competent cells (Promega, UK). Plasmids were isolated using the Qiafilter Plasmid Maxi Kit (Qiagen, UK), DNA concentrations were measured spectrophotometrically (Amersham, UK), and the number of *HPRT1*- and *CARD9*-copies/µL plasmid DNA were calculated. Constructs were examined by sequencing and restriction enzyme digestion, and 10-fold serial dilutions of the plasmids were prepared and stored at -20°C.

Total RNA extracts of different cell types and PBMCs were prepared using the RNeasy Minikit (Qiagen, UK) and subsequent reverse transcription was carried out using Omniscript reverse transcriptase (Qiagen, UK) after a precedent DNAse (Thermo Fisher, UK) digestion of isolated RNA samples according to the manufacturer's instruction.

SYBR® Green–based quantitative real-time PCRs (Qiagen, UK) for cDNA were performed in 20-µl capillaries on a LightCycler instrument (Roche, UK) by using *HPRT1* primers as described above and the following *CARD9* primers: CARD9-Fw 5-CCT GGT GTG TCT GCA GTG-3 and CARD9-Rv 5-CTC GTC ATC GTT CTC GTA GTC-3 (all primers were purchased from Invitrogen, UK). Cycling conditions consisted of an initial activation of 15 min at 95°C, 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, followed by melting curve analysis to identify amplification of the correct PCR product. The number of *CARD9* transcripts was determined according to standard curves and normalized to the number of *HPRT1* transcripts, which had been determined to standard curves as well. All primers used in this study were intron spanning, as demonstrated by the absence of amplification from genomic DNA.

Screening for Q295X mutations by restriction enzyme digestion

A 550 bp PCR product of exon 6 was amplified in heterozygous and homozygous healthy family members as well as in healthy controls by using the following PCR primers: Ex6-Fw 5-ACC TTC TGC AGA TTG ACC AG-3 and Ex6-Rv 5-CAG GAG TGG GTG AGT GGA-3. After cleaning up using the Qiagen PCR purification kit (Qiagen, UK), the obtained amplicons were diluted 1:4 and subjected to digestion by using the restriction enzyme Bfa1 (New England Biolabs, UK). As a control, PCR products of homozygous affected and heterozygous healthy individuals were digested, thereby revealing two fragments of 350 and 200 bp (homozygous affected, two alleles with one cutting site each) or three fragments of 550 bp, 350 bp and 200 bp (Heterozygous healthy, one allele with one cutting site, one without). PCR products of homozygous healthy individuals showed the original undigested PCR product.

T cell phenotyping and detection of Th17 cells by flow cytometry

Blood was collected from the four homozygous mutated patients, three heterozygous and four homozygous healthy relatives, peripheral blood mononuclear cells (PBMCs) were isolated by using LymphoprepTM (Axis-Shield, Norway) and subsequently viably frozen. Freshly thawed cells were resuspended at a concentration of 10^6/mL in RPMI (Lonza, UK) supplemented with 10% fetal calf serum (FCS, Gibco, UK).

T cell phenotyping was performed using the following antibodies: anti-CD3 (PE-Cy5-labelled), anti-CD45RO (APC), anti-CD45RA (APC), anti-CD27 (FITC), anti-CD28 (PE), anti-CD31 (PE), anti-TCR$\alpha\beta$ (PE) (all antibodies were purchased from Becton, Dickinson & Company (BD), UK), anti-CD4 (FITC), anti-CD8 (APC; both antibodies from EuroBioscience, U.K.) and anti-CXCR5 (PE; R&D Systems, UK).

Tregs were stained by using anti-CD4-(PerCP), anti-CD25-(PE) and anti-CD127-(FITC) antibodies according to a protocol previously published (all antibodies were purchased from BD Biosciences, UK).[34,35] In our samples, staining for CD127 worked more effectively than staining for the more widely used FoxP3 marker; expression of FoxP3 is negatively correlated with the expression of CD127.[34] Analysis was carried out on a FACSCalibur using the CellQuest software (BD, UK).

For detection of Th17 cells, thawed cells were exposed to Staphylococcus enterotoxin B (SEB; Sigma, US) 1µg/mL and Brefeldin A (Sigma, US) 2.5µg/mL for 16 hours at 37°C and 5% CO_2. The SEB stimulation served to assess the physiological cytokine production of an already committed T cell. A 16 h SEB stimulation does not drive naive T cells into the Th17 cell differentiation,[36] but merely makes visible already IL-17 committed Th17 cells and thus serves as a tool to assess Th17 cell numbers *ex vivo*.

The cells were then surface-stained for CD4 (PerCP) and CD45RO (PE-Cy7), followed by an intracellular staining for IL-17 (Alexa Fluor® 647) and IFN-γ (FITC). Intracellular staining was performed by using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, UK). All antibodies were from BD Biosciences, except for IL-17 (eBioscience, UK). Data were acquired on an LSR II flow cytometer and analyzed using FACSDiva software (BD Biosciences, UK). Wilcoxon rank-sum tests were performed with Prism 5.01 software (Prism, US); P-values are two-sided.

Immunoblot

Cell extracts of CARD9-deficient patients' PBMCs and PBMCs of unrelated healthy donors, homo- or heterozygous healthy family members were prepared by using cell lysis buffer (Cell Signaling, UK) supplemented with 1 mM PMSF, and after separation with a 10% SDS-PAGE, blotted and stained with a polyclonal goat anti-CARD9 antibody (Santa Cruz, US) or a polyclonal rabbit anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibody (R&D, UK). After staining with either an HRP-conjugated rabbit anti-goat (Cell Signaling, UK) or goat anti-rabbit antibody (R&D, UK) images were captured by using ECL chemiluminescence (GE Healthcare, UK) on Amersham Hyperfilms (Amersham, UK).

Author contributions

BG designed the study and decided to publish the paper, EG and JR designed aspects of the study. EG and AH gathered and analyzed the data with BG. MN and NR sign responsible for the clinical assessment and the patient sample collection and identity. FT organized the FBC and the general laboratory tests on the samples, SJ did the T cell phenotyping, CW performed the Th17 analysis and AM performed the

Treg cell analysis. US, DP and HV performed the genotyping, AAS performed the linkage analysis and compiled the list of positional candidate genes. KW was involved in picking the correct candidate. AAA provided the cohort of healthy Iranian controls, AP ruled out mutations in *AIRE*, and NH, OG and JR sign responsible for the genetic correction of the human defect in mice. All other results were produced by AH and EG. AH, EG, AAS, JR and BG wrote the paper. There are no agreements concerning confidentiality of the data between the sponsor and the authors or the institutions listed in this paper.

Results

Blood cell counts and T cell phenotyping

Neutrophils and $CD4^+$ helper T cells are critical in the host defense to candida species. We therefore studied the patients' complete blood counts and lymphocyte subsets. Total $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells, memory T cells ($CD45RO^+$), follicular helper T cells ($CD45RO^+/CXCR5^+$), effector memory T cells ($CD27^-/CD28^-$), regulatory T cells ($CD4^+/CD25^+$/CD127low), B cells ($CD19^+/CD45^+$) and natural killer (NK) cells ($CD16^+/CD45^+/CD56^+$) were all within the normal range in patients 1M, 2M and 5F (Supplementary Table S1). Furthermore, no significant alterations in basal serum immunoglobulin levels were detected (data not shown).

Genetic Linkage analysis

Analysis of the 250k Affymetrix chip data on 13 samples, including the five living patients did not show any perfect segregation of the CMC phenotype with an interval of at least 500kb length. Susceptibility to fungal infections is common and four of the affected individuals are more severely affected than 1B1.[8] Therefore, for each set of four out of five affected individuals, we reanalyzed the SNP data under the revised

assumption that four affected individuals are homozygous for the same haplotype and the fifth is a phenocopy. This analysis revealed one perfect region at the telomeric end on chromosome 9, spanning approximately 137.5-138.8Mbp. The more severe affected individuals 1M, 2B2, 2M, 5F shared the same homozygous haplotype, but the mildly affected 1B1 was heterozygous.

To confirm the SNP-derived evidence of linkage and compute LOD scores, we genotyped four polymorphic microsatellites in this region. D9S2157 (135.0Mbp) and D9S1818 (139.8Mbp) are outside the SNP-predicted interval. D9S1826 (137.6Mbp) and D9S158 (138.2Mbp) are inside the SNP-predicted linkage interval. D9S1826 is more informative in this pedigree and gave a peak LOD score of 2.37 at a recombination fraction of 0. A two-marker analysis using the more informative D9S1826 and D9S1838 peaked at 3.28. A three-marker analysis including D9S158 boosted the peak LOD score to 3.61, and the score is at least 3.59 for any placement of the gene between the markers.

CARD9 mRNA levels in various cell types

To determine the expression of human *CARD9,* we quantified *CARD9* messenger RNA (mRNA) of various cell types by real-time PCR and normalized the obtained values to *HPRT1* mRNA. Average levels of *CARD9* mRNA were highest in monocytes followed by granulocytes, B- and T cells, and the colon cell line HT-29 (Supplementary Figure 1). This expression pattern reflects the tissue distribution of murine *Card9*.[39] By examining PBMCs of patients with the homozygous *CARD9* mutation, we found that these cells still exhibit substantial levels of mutated *CARD9* mRNA (Supplementary Figure 1).

Functional consequences of CARD9 Q295X on signal transduction

To study the functional relevance of the CARD9 Q295X mutation on signal transduction, we cloned the human *CARD9* cDNA containing the Q295X mutation into a retroviral expression vector that can co-expresses green fluorescent proetein (GFP) from an internal ribosomal entry site (IRES). Using this construct, we retrovirally transduced CARD9 (Q295X) into primary bone marrow cells from Card9-deficient mice and in parallel transduced Card9-/- bone marrow with a control virus expressing only GFP or with a virus that co-expresses wild type human CARD9 and GFP (Supplementary Figure 2). The bone marrow cells were then differentiated into macrophages *in vitro* and analyzed by flow cytometry. Approximately 60% of the cultured macrophages expressed the constructs (Supplementary Figure 3).

2. Supplementary tables

Supplementary Table S1:

Blood counts and lymphocyte subsets of the four patients with CMC.

	Patient 2M	**Patient 1M**	**Patient 5F**	**Patient 2B**	**reference range**
white blood cells [10^3/µl]	6.01	6.85	5.24	7.63	4-10
Neutrophils [10^3/µl]/%	3.19/53	4.28/62	2.83/54	3.75/49.1	1.83-7.25/30-80
Monocytes [10^3/µl]/%	0.34/5.7	0.29/4.2	0.34/6.5	0.48/6.3	0.09-0.6/1-12
Eosinophils [10^3/µl]/%	0.18/3.0	0.09/1.3	0.09/1.7	1.06/13.9	0.08-0.36/0-6
Basophils [10^3/µl]/%	0.03/0.5	0.01/0.1	0.02/0.4	0.02/0.3	0.02-0.08/0-2
Lymphocytes [10^3/µl]/%	2.27/37.8	2.18/31.8	1.96/37.4	2.32/30.4	1.5-4/15-50
$CD3^+$ T Lymphocytes, absolute counts	1781	2056	1804	1951	690-2540
$CD3^+$ $CD45^+$ T Lymphocytes, percentage	71%	79%	76%	71%	55-84%
$CD3^+/CD4^+/CD45^+$ T Lymphocytes, absolute counts	1096	1430	1070	1139	410-1590
$CD3^+/CD4^+/CD45^+$ T Lymphocytes, percentage	42%	54%	47%	42%	31-60%
$CD3^+/CD8^+/CD45^+$ T Lymphocytes, absolute counts	640	604	611	628	190-1140
$CD3^+/CD8^+/CD45^+$ T Lymphocytes, percentage	24%	23%	27%	23%	13-41%
$CD8^+$ T cells, percentage	19%	23%	32%	n. d.	16-31%
$CD45RO^+$ T cells, percentage	44%	63%	51%	n. d.	38-72% of $CD4^+$
$CD45RO^+$ $CXCR5^+$ so called ‘T follicular helper cells’, percentage	15%	9%	8%	n. d.	6-16% of $CD4^+$
$CD27^-$ $CD28^-$ cells, so called ‘late effector T cells’, percentage	30%	47%	27%	n. d.	4-32% of $CD8^+$
$CD27^+$ $CD28^-$, so called ‘effector memory T cells’, percentage	13%	6%	18%	n. d.	1-24% of $CD8^+$
double negative T cells ($CD3^+/CD4^-/CD8^-$), percentage	<1%	<1%	<1%	n. d.	4% (1-10)

$CD31^+/CD45RA^+$ T cells, thought to represent recent thymic emigrants, percentage	58%	81%	62%	n.d.	48-76%
$CD4^+/CD25^+$/CD127low T cells, thought to be regulatory T cells, percentage	3.97%	2.90%	2.88%	n. d.	2-7%
$CD16^+/CD56^+$ /$CD45^+$ NK cells, absolute counts	317	122	129	379	90-590
$CD16^+/CD56^+/CD45^+$ NK cells, percentage	13%	5%	5%	13%	5-27%
$CD19^+/CD45^+$ B Lymphocytes, absolute counts	373	391	433	405	90-660
$CD19^+/CD45^+$ B Lymphocytes, percentage	15%	15%	17%	14%	6-25%

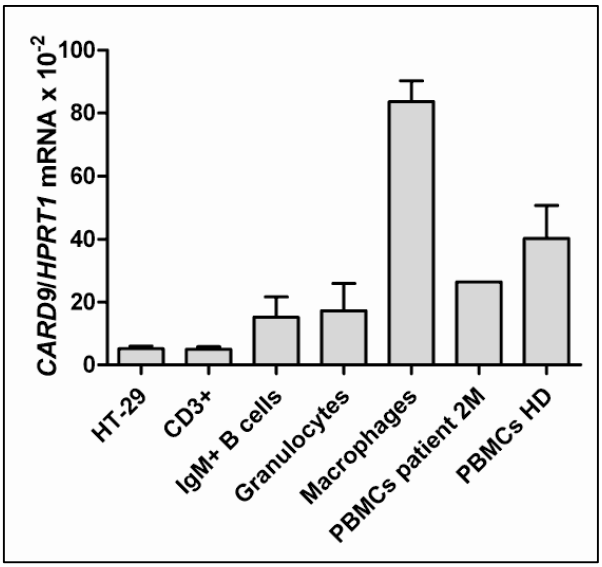
Supplementary Table S2:

Genes in the approximately 1.3Mb minimal linkage interval suggested by the SNP data, as of February 2008, when the hypothesis that the mutated gene is *CARD9* was first considered. Subsequent improvements in gene prediction caused some predicted genes (starting in LOC) to be added and others to be dropped, and caused *SNORA17* to be dropped.

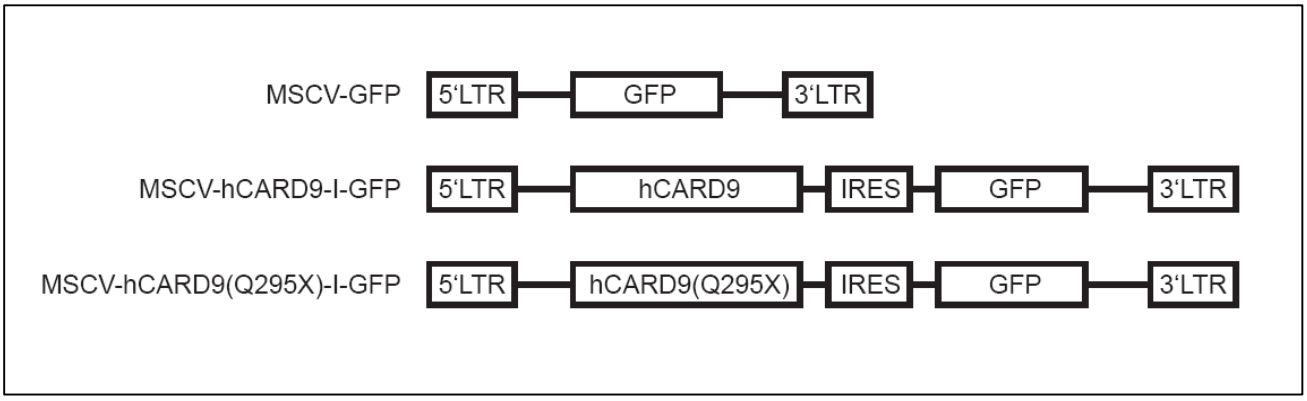
1	*KIAA0649*
2	*C9orf116* chromosome 9 open reading frame 116
3	*MRPS2* mitochondrial ribosomal protein S2
4	*LCN1* lipocalin 1 (tear prealbumin)
5	*OBP2A* odorant binding protein 2A
6	*LOC654089* predicted gene
7	*PAEP* progestagen-associated endometrial protein
8	*GLT6D1* glycosyltransferase 6 domain containing 1
9	*LCN9* lipocalin 9
10	*SOHLH1* spermatogenesis and oogenesis specific basic helix-loop-helix 1
11	*KCNT1* potassium channel, subfamily T, member 1
12	*CAMSAP1* calmodulin regulated spectrin-associated protein 1
13	*UBAC1* UBA domain containing 1
14	*NACC2* NACC family member 2, BEN and BTB (POZ) domain containing
15	*LOC728436* predicted gene
16	*LOC402382* predicted gene
17	*C9orf69* chromosome 9 open reading frame 69
18	*LOC728446* predicted gene
19	*LOC643641* predicted gene
20	*LHX3* LIM homeobox 3
21	*QSOX2* quiescin Q6 sulfhydryl oxidase 2
22	*GPSM1* G-protein signaling modulator 1 (AGS3-like, C. elegans)
23	*DNLZ* DNL-type zinc finger
24	*CARD9* caspase recruitment domain family, member 9
25	*SNAPC4* small nuclear RNA activating complex, polypeptide 4, 190kDa
26	*SDCCAG3* serologically defined colon cancer antigen 3
27	*PMPCA* peptidase (mitochondrial processing) alpha
28	*INPP5E* inositol polyphosphate-5-phosphatase, 72 kDa
29	*SEC16A* SEC16 homolog A (S. cerevisiae)
30	*C9orf163* chromosome 9 open reading frame 163
31	*NOTCH1* Notch homolog 1, translocation-associated (Drosophila)
32	*EGFL7* EGF-like-domain, multiple 7
33	*MIR126* microRNA 126
34	*AGPAT2* 1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta)
35	*FAM69B* family with sequence similarity 69, member B
36	*SNORA43* small nucleolar RNA, H/ACA box 43
37	*SNORA17* small nucleolar RNA, H/ACA box 17
38	*LCN10* lipocalin 10
39	*LCN6* lipocalin 6
40	*LCN8* lipocalin 8

41	*RP11-216L13.5* MSFL2541 (a.k.a. UNQ2541)

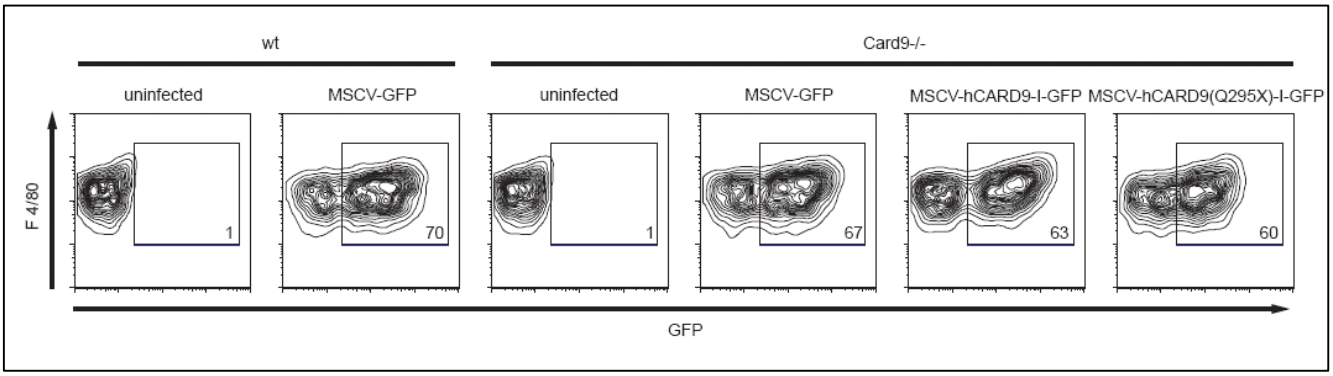
3. Supplementary figures



Supplementary Figure 1: *CARD9* expression in various human cell types: HT-29 cells (colon cell line), $CD3^+$ T cells, B cells, granulocytes, macrophages and PBMCs of healthy donors. RNAs of cells were isolated, transcribed into complementary DNA (cDNA) and then subjected to real-time PCR as described in methods. In order to make samples comparable, *CARD9* values were normalized to the expression of a housekeeping gene (Normalization to *HPRT1*). Results represent an average of three independent experiments (error bars, s. d.) except for patient 2M, where only a single sample was tested.



Supplementary Figure 2: Schematic representation of the retroviral constructs that were used to transduce primary bone marrow cells from wild type or *Card9*-/- mice.



Supplementary Figure 3: Retroviral gene expression in Card9-/- macrophages. Bone marrow cells from wt or *Card9*-/- mice were left untransduced or transduced with MSCV-GFP, MSCV-hCARD9-I-GFP or MSCV-hCARD9(Q295X)-I-GFP, differentiated into macrophages and subsequently analyzed by FACS for GFP and F4/80 expression. The percentage of retrovirally transduced F4/80 positive macrophages in the cell culture is indicated.